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CONTRACT NO: MIPR 2505

TITLE: PREPARATION AND CHARACTERIZATION OF MOUSE AND HUMAN MONOCLONAL ANTIBODIES TO BOTULINUM TOXINS

PRINCIPAL INVESTIGATOR: Kenneth W. Hunter
Gerald W. Fisher
Val G. Hemming

CONTRACTING ORGANIZATION: Uniformed Services University of Health Sciences
Department of Pediatrics
4301 Jones Bridge Road
Bethesda, MD 20814

REPORT DATE: January 1983

TYPE OF REPORT: Progress Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) PREPARATION AND CHARACTERIZATION OF MOUSE AND HUMAN MONOCLONAL ANTIBODIES TO BOTULINUM TOXINS	5. TYPE OF REPORT & PERIOD COVERED Progress Report
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Kenneth W. Hunter, Gerald W. Fisher, Val G. Hemming	8. CONTRACT OR GRANT NUMBER(s) MIPR 2505
9. PERFORMING ORGANIZATION NAME AND ADDRESS Uniformed Services University of Health Sciences Dept. of Pediatrics, Bethesda, MD 20814	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, MD 21701	12. REPORT DATE January 1983
	13. NUMBER OF PAGES 16 pages
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

mice, human monoclonal antibodies, enzyme immunoassays, type B toxoids, type F toxoids, toxic, [illegible], Botulinum Toxins

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The goal during this year of the project was to begin preparing and characterizing mouse and human monoclonal antibodies to botulinum toxoids. The initial step was to refine enzyme immunoassays for identifying both mouse and human monoclonal antibodies in hybridoma culture supernatants. Progress in this area has been good, though minor technical problems remain to be solved. Optimization of these assays was accomplished with hyperimmune mouse and human antisera obtained from Dr. Martin Crumrine, USAMRIID,

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FOREWORD

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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1. BRIEF RESEARCH SYNOPSIS

The goal during year 01 of this project was to begin preparing and characterizing mouse and human monoclonal antibodies to botulinum toxoids. The initial step was to refine enzyme immunoassays for identifying both mouse and human monoclonal antibodies in hybridoma culture supernatants. Progress in this area has been good, though minor technical problems remain to be solved. Optimization of these assays was accomplished with hyperimmune mouse and human antisera obtained from Dr. Martin Crumrine, USAMRIID.

We began to prepare mouse monoclonal antibodies against pentavalent botulinum toxoid. Hybridomas were prepared and their antibody products identified by enzyme immunoassay employing pentavalent toxoid as the antigen. Although more than a dozen positive culture supernatants were identified, none of these were frankly protective in the standard mouse protection assay performed at USAMRIID. As the pentavalent toxoid preparation contains only 10% toxoid by weight, it is likely that most if not all of the antibodies were directed to other protein species. We decided to screen these supernatants on monovalent types B and E toxoids. Some of the supernatants did react with type B, while none reacted with type E. However, the latter was never successfully employed as an antigen in the enzyme immunoassays using hyperimmune antisera. The type B toxoid results were at first encouraging, but the same problems with purity exist with these monovalent toxoid preparations.

These initial studies led us to the conclusion that we needed 1) to use highly purified antigen preparations, and 2) to immunize mice with single toxoid types rather than pentavalent toxoid. As the present project was limited to the preparation of hybridomas but not botulinum toxoids, we had a short moratorium while highly purified toxins A and B were obtained from Dr. Das Gupta, and these toxins toxoided by Dr. Crumrine at USAMRIID.

Upon receiving these toxoids, we attempted to establish enzyme immunoassays. Unfortunately, only the type B material proved viable; the type A toxoid was apparently denatured. This finding was also documented by Dr. Crumrine. We immunized mice with type B and prepared hybridomas. Seven high titered cultures were identified by enzyme immunoassay against type B toxoid and samples of culture supernatants sent to USAMRIID for mouse protection tests. None of these supernatants were protective. Nevertheless, the use of pure toxoid strongly suggests that these antibodies are indeed directed to botulinum toxoid type B epitopes. Failure in the protection assay could have resulted from 1) antibody reactivity against toxoid, but not toxin epitopes, or 2) from failure to isolate antibodies that bind to the "toxic" site on the toxin molecule. The findings suggest that a multitude of mouse monoclonal antibodies may have to be screened to identify one or two with toxin-neutralizing capabilities. However, should the site(s) on the toxin molecule responsible for binding to receptors or internalization be identified by other contractors, these fragments could be used as immunogens to increase the likelihood of finding toxin-neutralizing antibodies.

Notwithstanding the early discouraging results with toxin neutralization, the antibodies isolated so far may certainly find utility as diagnostic reagents. In the next segment of our contract, as more purified toxoids are made available from USAMRIID, we will determine the cross-reactivity of each antibody for the various toxoid types using the enzyme immunoassay system.

It should be noted that all of the hybridomas identified as anti-botulinum toxoid antibody secretors have been expanded in culture and cryopreserved. Two of the type B reactive hybridomas have been cloned and the clones expanded in numbers and cryopreserved.

A brief word is in order about the human hybridoma portion of the project. Although we have concentrated on mouse hybridomas in the initial phases of the project, some work has been done on human hybridomas. We have obtained peripheral blood lymphocytes from a USAMRIID donor at several time points following an annual booster immunization with pentavalent toxoid. Although one aborted attempt was made to prepare hybridomas, the majority cells have been cryopreserved for use at a later time. As the human lymphocytes are a limited resource, we plan to perfect the assays and hybridoma procedures in the mouse system before exerting a concentrated effort toward preparing human hybridomas. However, some initial fusions are planned for early in the spring of 1983.

We conclude this brief summary by pointing out that progress in this contract is somewhat dependent on the supply of purified botulinum toxoids. It may be fortuitous, however, that the supply of toxoids is limiting, as we may be required to produce and screen hundreds of monoclonals to each toxoid type to find one that neutralizes. Over the next few months we plan to adapt the existing cloned hybridomas to growth as ascites tumors in mice so that large quantities of antibody can be produced. We will supply workers at USAMRIID with these antibodies to facilitate the development of a standardized and reliable immunoassay for botulinum toxin, and to facilitate studies of the structure-function relationships of botulinum toxins.

2. DETAILED PROGRESS REPORT

2.1. Enzyme immunoassays for mouse and human anti-botulinum toxoid antibodies

A reliable and reproducible assay for detecting monoclonal antibodies is essential to the hybridoma technique. As this project involved the preparation of both mouse and human monoclonal antibodies reactive with botulinum toxoids, we developed appropriate enzyme immunoassays.

2.1.1. Enzyme immunoassay using pentavalent botulinum toxoid vaccine

Serum from a mouse hyperimmunized with pentavalent toxoid (types A-E) was used to standardize the mouse assay. The solid-phase adsorbant employed was a round-bottom polystyrene microtiter plate (Dynatech, Alexandria, VA). Pentavalent toxoid (Michigan Dept. of Public Health, lot A2) was tested at various dilutions in 0.1 M carbonate buffer, pH 9.6. The optimum dilution (1:100) was added in volumes of 100 µl to the plates and allowed to incubate at 4°C overnight. The plates were then washed with phosphate buffered saline containing 0.05% Tween 20 (PBS-T) by 5 cycles of filling and emptying each well. Various dilutions of mouse antiserum in PBS-T were added in 100 µl volumes to appropriate wells and allowed to incubate for 30 min at 4°C. The plates were washed as described above, and 100 µl of an appropriate dilution of rabbit anti-mouse kappa (light chain) was added for 30 min at 4°C. After another wash cycle, 100 µl of goat anti-rabbit IgG conjugated with the enzyme alkaline phosphatase was added. Thirty min later the plate was washed and 100 µl of p-nitrophenylphosphate (substrate, 1 mg/ml) in 10% diethanolamine buffer, pH 9.8, was added. After 30 min the yellow colored product (p-nitrophenol) of the enzyme-substrate reaction was measured spectrophotometrically. A Flow Titertek Multiscan micro-ELISA reader was used to scan the plates at 405 nm. Controls included normal mouse serum substituted for the mouse hyperimmune serum, and the later incubated in wells not coated with antigen.

A representative titration curve is shown in Fig. 1. Under the conditions described above, the mouse hyperimmune serum gave a fairly linear titration curve from undilute to a dilution of approximately 1:10,000. The absorbance generated by normal mouse serum and the mouse antiserum on uncoated wells was less than 0.1 units. It should be noted that use of the anti-kappa reagent in this assay effectively detects all mouse immunoglobulin classes and subclasses since 98% of mouse immunoglobulins bear the kappa rather than the lambda light chain. Also, the double antibody procedure amplifies the signal since multiple enzyme-conjugated goat antibodies bind to each rabbit antibody.

A very similar procedure was used for measuring human antibodies to pentavalent toxoid. Serum from an individual that had received a series of immunizations with the pentavalent toxoid was employed as the standard. As in the mouse assay, a double-antibody method was used. The only difference in the assay was the use of a heavy chain specific rabbit anti-human IgG reagent. We felt that the immunization scheme used in the donor would most likely result in IgG rather than IgM anti-toxoid antibodies. It was understood, however, that a reagent that could detect both IgG and IgM antibodies (e.g., rabbit anti-human immunoglobulin or a mixture of the IgG and IgM-specific reagents) would be employed for screening the human-human hybridomas.

A representative titration curve for this assay is shown in Fig. 2. A linear titration curve was also obtained with the human antiserum.

2.1.2. <u>Enzyme immunoassay using monovalent types B and E botulinum toxoids</u>

The first monovalent toxoids that were made available by USAMRIID were types B and E. We attempted to use these toxoids as we used the pentavalent toxoid in an enzyme immunoassay. It should be noted that the quantities of these toxoids were limited, and the concentrations used to coat plates were the highest levels we could afford to use.

To assess the assay using these purified toxoids, we employed the plasma from our human donor. Types B and E toxoids were diluted to 1:100 in coating buffer and allowed to incubate in the microtiter wells overnight at 4°C. Pentavalent toxoid was used as described above.

The results from this experiment are shown in Fig. 3. Note that the donor plasma titrated best on the pentavalent toxoid, a finding not too surprising since presumably the plasma contains antibodies specific for all 5 toxoid types. A reasonably good titration curve was also seen with the type B toxoid, but very little activity was observed on type E toxoid. A normal human serum control yielded a titration curve superimposable on the toxoid E curve. These observed differences might be due to any of the following: i) the human plasma contains very little anti-toxoid E, ii) the absolute concentration of toxoid E is lower than toxoid B, iii) inherent differences in toxoids B and E result in different adsorption rates to polystyrene, iv) toxoid E contains some blocking factor, or v) toxoid E has been denatured. These possibilities are not mutually exclusive. In addition, Dr. Martin Crumrine, USAMRIID, noted the same problem with E toxoid in a different mode of immunoassay.

2.2. Preparation of mouse monoclonal antibodies to pentavalent botulinum toxoid

In the first set of immunizations, donor mice were given 0.2 ml of pentavalent toxoid SC in Freund's complete adjuvant followed at 14 days by the same amount IP in saline. Three days later the spleens of two donors were removed and the dissociated lymphocytes (10^8) fused with mouse plasmacytoma P3-X63-Ag8.653 (10^7). At two weeks the culture supernatants were screened against pentavalent toxoid, and 14/192 wells were positive (EIA absorbance values greater than 4 times background). The cultures were expanded to 24 well plates and supernatants rescreened 7 days later. At this time only 8 of the 14 positives continued to secrete. All 8 were expanded to flask culture, supernatants harvested over 7-10 days, then the cells were cryopreserved.

Supernatants from 13 of the original 14 positives were tested for antibody reactivity against monovalent type B and E toxoids. These results are shown in Table 1. All 13 supernatants reacted with the pentavalent toxoid, whereas only 7 showed reactivity to the type B toxoid. None of the supernatants demonstrated binding to type E toxoid, but this result is probably due to the very poor quality of this toxoid.

Supernatants from the 8 hybridoma cultures with stable antibody production after 1 month were sent to Dr. Martin Crumrine, USAMRIID, for testing in the standard WHO mouse protection assay. The results of these studies were equivocal. These supernatants were also tested in a competitive binding assay by Dr. Crumrine; two of the supernatants gave weakly positive results with type B toxin.

One highly positive hybridoma designated B-C12 was cloned by limiting dilution on irradiated mouse tumor macrophages (P388D1). Positive clones were identified by EIA on pentavalent toxoid, expanded in number, and cryopreserved.

2.3. Preparation of mouse monoclonal antibodies to purified botulinum toxoid type B

In a second set of immunizations, donor mice were immunized SC with 0.2 ml of highly purified botulinum toxoid type B emulsified in Freund's complete adjuvant. At 14 days, the mice were boosted with another 0.2 ml given IP. Three days later spleens were removed and the splenic lymphocytes dissociated into single cell suspensions. These cells were fused exactly as described earlier. At 14 days post-fusion supernatants of these cultures were tested for antibody reactivity against type B toxoid; 32 of 288 wells (11%) were positive by EIA. These positives were expanded to larger wells and 16 with active growth tested two weeks later. At this time only seven were still actively secreting antibody (Table 2). Two of these were selected for cloning.

In the course of assaying the anti-B toxoid antibodies, we performed an experiment to determine whether purified B toxoid diluted in coating buffer could be "recycled." The prevailing opinion among USAMRIID workers was that the alkaline pH of coating buffer would denature the toxin, and possibly the toxoid, rendering it unusable for immunoassay. We tested 1:100 dilutions of type B toxoid harvested from polystyrene plates and held in coating buffer 2 weeks at 4°C against freshly prepared toxoid B. The result of this experiment are shown in Fig. 4. Note that overnight coating with recycled and fresh type B toxoid resulted in very similar and usable EIA titration curves. This finding is of particular importance given the limited amount of purified type B toxoid available. We now routinely harvest the coating antigen after its 24 hr incubation in the polystyrene plates and reuse it in later experiments.

TABLE 1

PRELIMINARY EVALUATION OF HYBRIDOMAS PREPARED FROM

A PENTAVALENT BOTULINUM TOXOID-IMMUNIZED MOUSE

	Test Antigen[1]		
Supernatant	Pentavalent	B	E
A-B11	1.45	0	0
A-D12	1.38	1.23	0
A-H1	1.20	0	0
B-A3	1.88	0	0
B-C12	>2.00	1.15	0
B-E12	1.35	0.55	0
B-H4	1.78	0	0
B-C8	>2.00	0.60	0
B-D8	1.68	0.26	0
B-G4	0.20	0	0
B-B1	0.38	0	0
A-G9	1.38	0.50	0
B-D12	1.30	1.00	0
Neg. Cont.	0.06		

[1] Numbers represent mean absorbance values of hybridoma supernatants in an enzyme immunoassay

TABLE 2

RESULTS FROM BOTULINUM TOXOID TYPE B FUSION

Fusion Well[a]	EIA (O.D. 405 nm)[b]	
A-C4	0.57[c]	POS[d]
A-D3	0.36	
A-E11	0.51	POS
A-H12	0.47	POS
B-B11	0.29	
B-D10	0.13	
B-F2	0.14	
B-F5	0.41	
B-F8	0.38	
B-H5	0.72[c]	POS
C-D4	0.51	POS
C-D7	0.15	
C-E4	0.59	POS
C-F2	0.05	
C-H6	0.25	
C-H12	0.12	
Medium Only	0.06	

[a] Retest of 16 wells with viable hybrids

[b] Mean O.D. of 2 replicates

[c] Cloned

[d] POS = positive, chosen for further processing

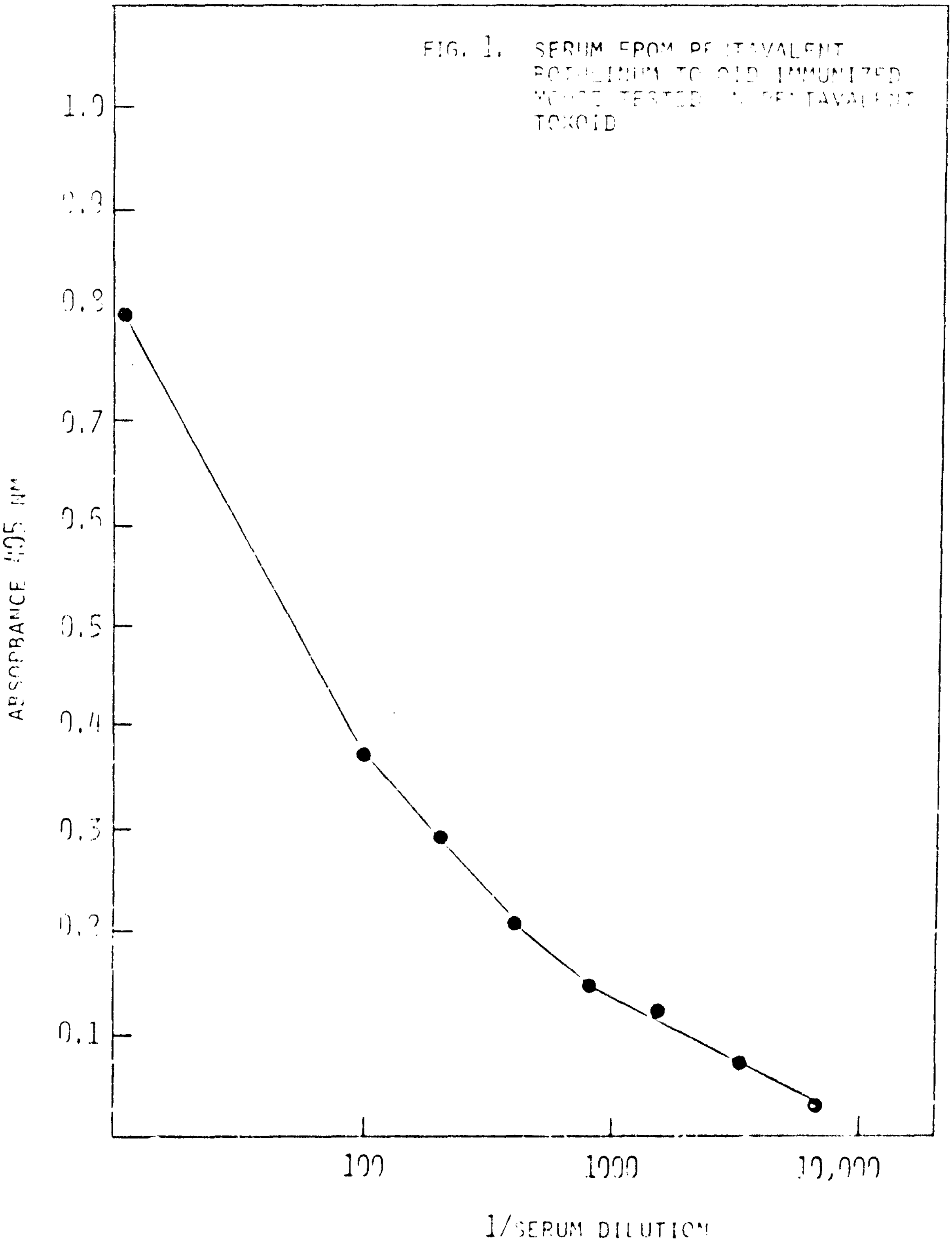

FIG. 1. SERUM FROM PENTAVALENT BOTULINUM TOXOID IMMUNIZED MOUSE TESTED ON PENTAVALENT TOXOID

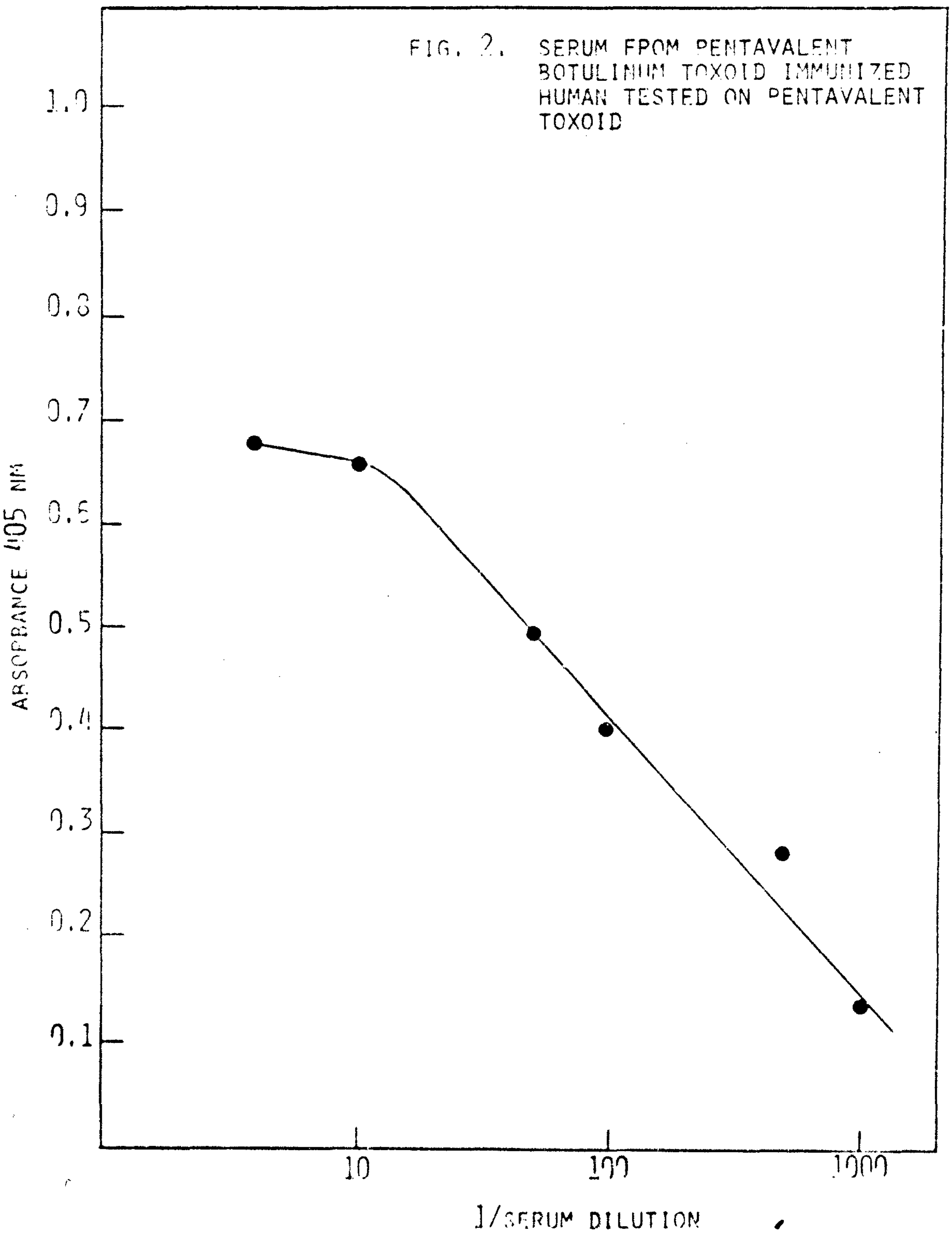

FIG. 2. SERUM FROM PENTAVALENT BOTULINUM TOXOID IMMUNIZED HUMAN TESTED ON PENTAVALENT TOXOID

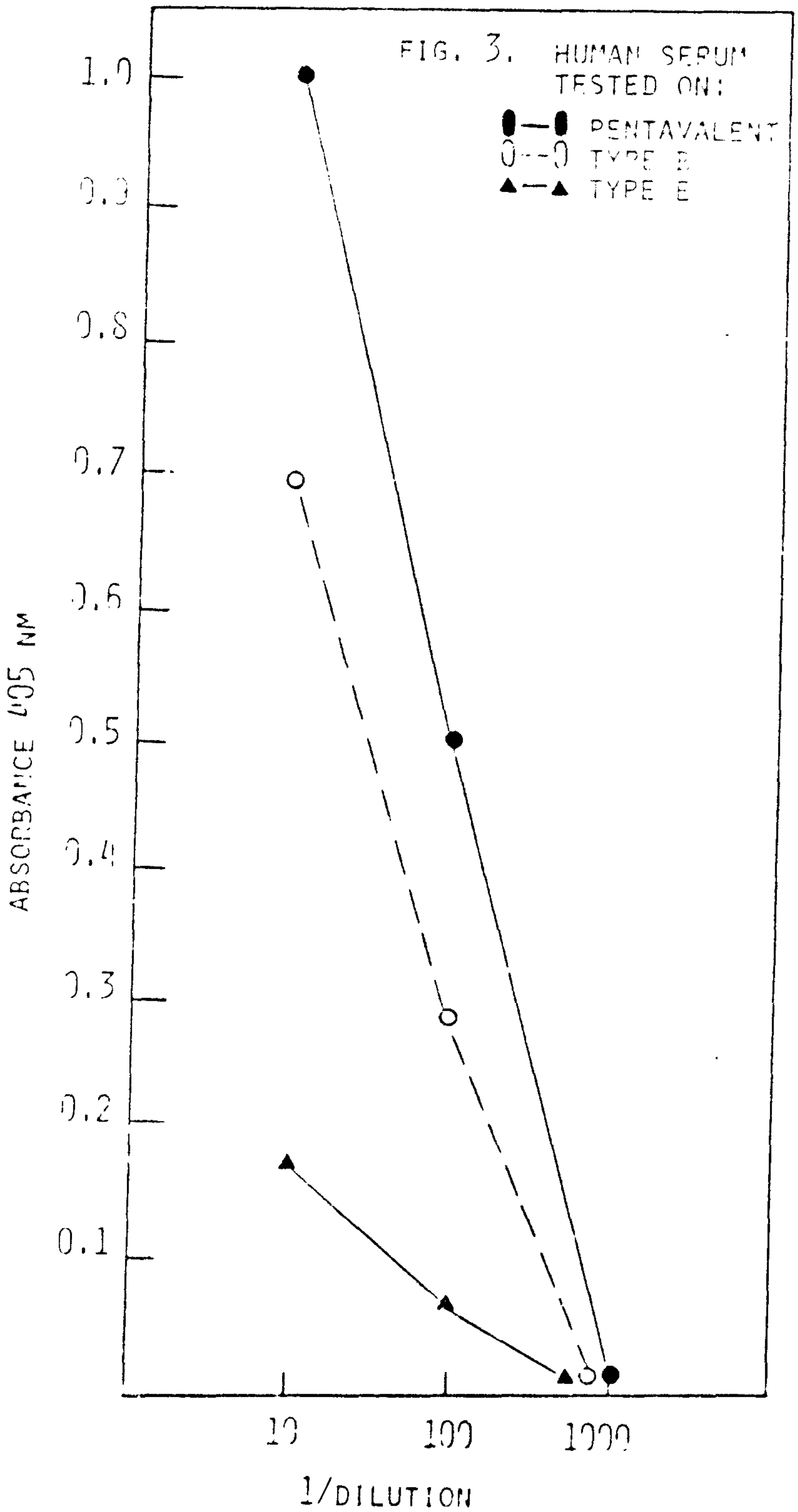

FIG. 3. HUMAN SERUM TESTED ON:
PENTAVALENT
TYPE B
TYPE E
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE 405 NM
10
100
1000
1/DILUTION

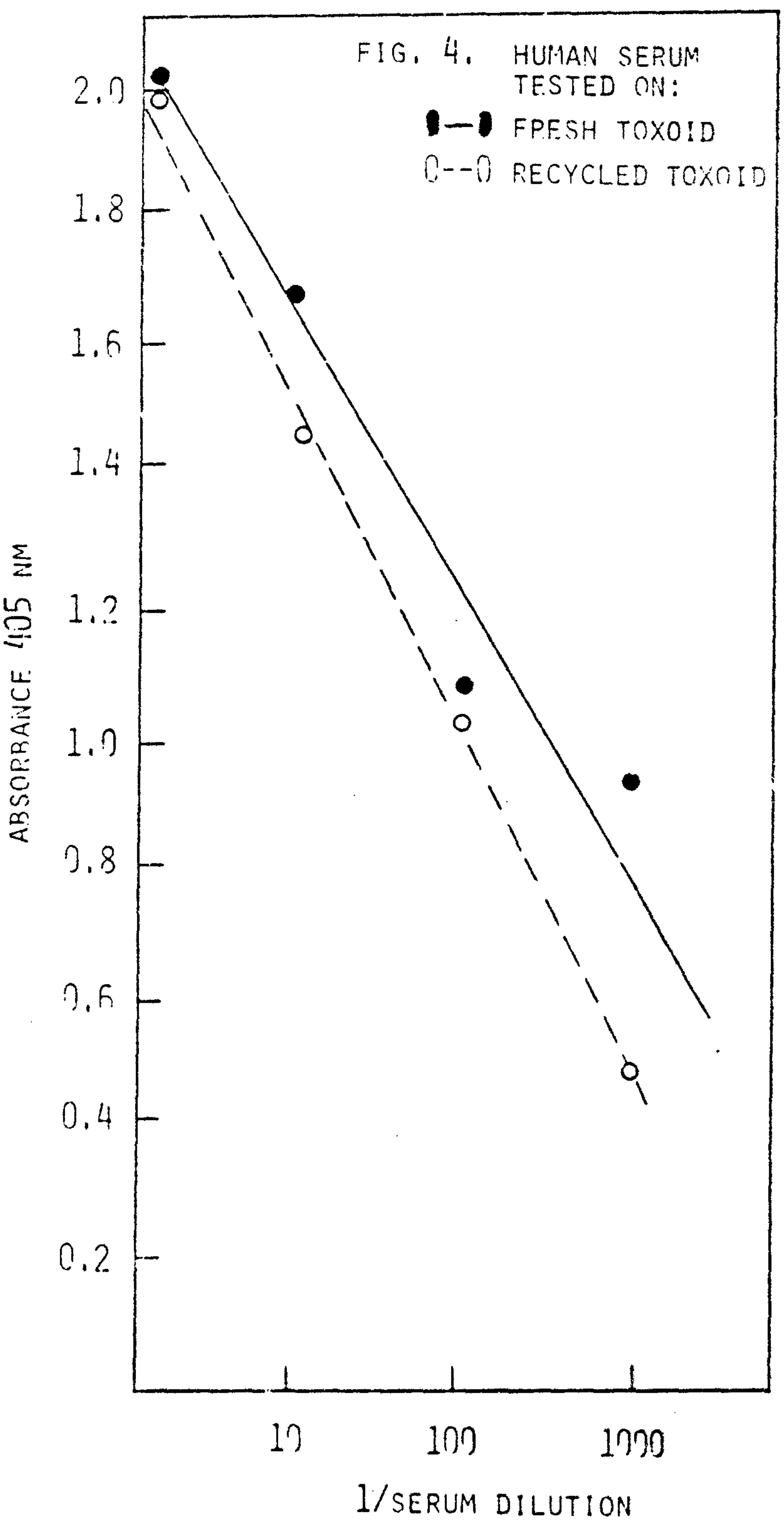

FIG. 4. HUMAN SERUM TESTED ON:
●—● FRESH TOXOID
O--O RECYCLED TOXOID
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE 405 NM
10
100
1000
1/SERUM DILUTION